Molecular Staging of Cutaneous T-Cell Lymphoma: Evidence for Systemic Involvement in Early Disease

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Biopsies of various tissues from eight patients with confirmed cutaneous T-cell lymphoma were analyzed for lymphomatous involvement using V-J junctional sequences in rearranged T-cell receptor-γ genes as specific molecular markers for the malignant clone. The patients included one stage IA, one stage IB, and six stage IVA. Twenty-five specimens were analyzed including 14 skin, five lymph node, four blood, and two bone-marrow samples. Ten skin samples and four lymph node samples were histologically positive for lymphoma. The other specimens were morphologically uninvolved. An assay involving polymerase chain reaction (PCR) amplification of T-cell receptor-γ gene rearrangements and denaturing gradient gel electrophoresis was used to identify the tissue specimen containing the greatest tumor clone density in each case. This specimen was then used to generate a tumor-specific RNA probe that was used to molecularly stage each patient by means of an assay involving PCR gene amplification and RNase protection analysis (PCR/RPA). This assay detected malignant cells in all available biopsies, including morphologically uninvolved extracutaneous tissue samples (two blood, one lymph node, and one bone marrow) obtained from the two patients in pathologic stage I. Microscopic examination and the less sensitive PCR/denaturing gradient gel electrophoresis technique failed to detect lymphomatous involvement in 11 (44%) and eight (32%) of these 25 specimens, respectively. We conclude that molecular biologic staging using PCR/RPA is able to demonstrate morphologically occult dissemination of cutaneous T-cell lymphona in early disease. In addition, PCR/RPA may be able to monitor tumor response to therapy and detect early recurrence of malignant lymphomas during clinical remission. *Key words: polymerase chain reaction/RNase protection/T-cell receptor gene rearrangements. J Invest Dermatol 104:889–894, 1995*

Lymphoid malignancies can be detected as a clonal excess of a specific antigen receptor gene rearrangement over polyclonal rearrangements by analysis of genomic DNA extracted from biopsy specimens with the Southern blot technique [1–7]. Although a Southern blot is labor intensive and requires DNA from at least 10^6 cells for one assay, the recent introduction of amplification of rearranged antigen receptor genes by the polymerase chain reaction (PCR) allows analysis of extremely small samples [2]. Previously, we described the rapid detection of clonal T-cell receptor γ chain (TCR-γ) gene rearrangements by the combination of PCR and denaturing gradient gel electrophoresis (PCR/DGGE) as a diagnostic criterion for lymphoid neoplasms [8,9]. In addition, we have developed a PCR-based RNase protection assay (PCR/RPA) for the specific detection of the individual malignant clone in acute lymphoblastic leukemia by its TCR-γ gene rearrangement [10]. This assay is able to detect a single malignant cell among 10^5 normal bone marrow cells.

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Abbreviations: DGGE, denaturing gradient gel electrophoresis; RPA, RNase protection analysis.

In this study, we have tested the hypothesis that the detection of malignant lymphocytes with molecular assays of high sensitivity might be a significant improvement for the staging of a solid lymphoid malignancy. We decided to focus on the analysis of cutaneous T-cell lymphoma (CTCL) for several reasons. First, the stage of CTCL at the time of diagnosis clearly has prognostic significance for both the response to therapy and survival of the patients [11]. In addition, the analysis of TCR gene rearrangements is a well established procedure for this type of malignant lymphoma [12,13]. Third, skin biopsies with a scant lymphoid infiltrate are often seen in this disease and frequently do not yield sufficient DNA for Southern blot analysis [9,11]. Finally, we had access to a well characterized group of patients with CTCL whose diagnostic biopsies had already been analyzed for clonal TCR-γ gene rearrangements by PCR/DGGE. We now describe the molecular detection of lymphomatous involvement in a variety of cutaneous and extracutaneous biopsy specimens from these patients and compare the use of the aforementioned assays with conventional diagnostic methods.

MATERIALS AND METHODS

Case Selection Eight patients from a previous study investigating the use of PCR/DGGE in the diagnosis of CTCL [9] had at least two different biopsies with remaining frozen material and were chosen for this study. All patients had histologically confirmed disease and clonal TCR-γ gene rearrangements as shown by PCR/DGGE. **Table I** summarizes the diag-

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Table I. Detection of Involvement by CTCL Using Histopathology, PCR/DGGE, and PCR/RPA Shows an Increasing Hierarchy of Sensitivity[a]

Patient (Stage)	Biopsy (Date)	Histopathology (Sensitivity, 56%)	PCR/DGGE (Sensitivity, 68%)	PCR/RPA (Sensitivity, 100%)
A (IV A)	Skin tumor (12/14/89)	MF	+	Probe
(T3N3M0B0)	Skin patch (12/14/89)	MF	+	1:500
	Lymph node (12/14/89)	MF	−	1:500
B (IV A)	Skin tumor (1/10/91)	MF	+	Probe
(T3N3M0B0)	Lymph node (1/10/91)	MF	+	1:100
C (IV A)	Skin patch 1 (2/21/90)	Chronic dermatitis	−	1:200
(T2N3M0B0)	Skin patch 2 (2/21/90)	Chronic dermatitis	−	1:500
	Lymph node (11/26/90)	MF	+	Probe
	Blood (3/20/90)	Normal	−	1:500
D (IV A)	Skin plaque (4/25/90)	MF	+	Probe
(T2N3M0B1)	Skin patch (4/25/90)	MF	+	1:5
E (IV A)	Skin plaque 1 (9/5/90)	MF	+	Probe
(T4N3M0B0)	Skin plaque 2 (1/2/91)	Chronic dermatitis (post Rx)	+	1:10
	Skin papules (11/28/90)	Chronic dermatitis (post Rx)	+	1:10
	Bone marrow (9/17/90)	Normal	+	1:10
	Blood (10/3/90)	Normal	+	1:10
F (IV A)	Skin tumor (1/14/91)	MF	+(Vγ1)/+(Vγ9)	Probe
(T3N3M0B0)	Lymph node (1/18/91)	MF	−(Vγ1)/+(Vγ9)	1:5000(Vγ1)/1:10(Vγ9)
G (IA)	Skin patch 1 (2/4/91)	MF	+	1:5
(T1N0M0B0)	Skin patch 2 (2/4/91)	MF	+	Probe
	Lymph node (2/4/91)	Normal	−	1:200
	Bone marrow (7/30/90)	Normal	−	1:100
	Blood (6/5/91)	Normal	−	1:100
H (IB)	Skin patch (6/19/91)	MF	+	Probe
(T2N0M0B0)	Blood (6/20/91)	Normal (rare atypical cells)	−	1:500

[a] MF, mycosis fungoides; Rx, Radiation therapy. + denotes clonal pattern on DGGE; − denotes polyconal smear on DGGE. Patient F was analyzed using clonal Vγ1 and Vγ9 rearrangements (see text). The results of the RNase protection assays are given as the relative intensity of the tumor-specific bands compared to logarithmic dilutions of DNA from the respective diagnostic biopsy into normal PBMC DNA. Staging based on criteria reviewed in [11].

nosis and pathologic stage at the time when the biopsies were performed, the type of tissue biopsied, and the date of the procedure. All patients were diagnosed with mycosis fungoides (MF), a subtype of CTCL that typically presents only with skin lesions [11]. The diagnosis of CTCL was established according to routine clinical and histopathologic criteria [11]. The definition of pathologic stages was taken from [11]. Because biopsies were generally performed only when extracutaneous disease was suspected clinically, the majority of patients tended to be in stage IV. However, no patient had extracutaneous manifestation of the disease by histopathologic criteria apart from involvement of lymph nodes in six patients and peripheral blood in one patient.

Samples All biopsies were performed with informed consent. A part of each specimen was processed for histopathologic examination. The remaining material was cryopreserved at −70°C until further processing. Normal peripheral blood was obtained from healthy volunteers. Mononuclear cells from blood and bone-marrow specimens were purified by Ficoll density gradient centrifugation. DNA was extracted from tissues using routine procedures [14].

PCR/DGGE The oligonucleotide primers used for PCR/DGGE were designed to amplify TCR-γ gene rearrangements involving Vγ1-9 and Jγ1-2 gene segments [15–18]. Rearranged TCR-γ genes were amplified from 1 μg of DNA using downstream primer Jγ1.2 (CGTCGACAACAAGTGTTGTTCCAC) and upstream primers Vγ1.2 (GAAGCTTCTAGCTTTCCTGTCTC) or Vγ9.1 (GGAATTCCAAATTCTTGGTTTA) as described previously [9]. Briefly, the first round consisted of 25 cycles each of which included DNA denaturation at 94°C for 1 min (3 min in the first cycle), primer annealing at 55°C for 1 min, and primer extension at 70°C for 1 min (6 min in the last cycle). Twenty percent of this reaction product was reamplified with nested downstream primer Jγint (GGATCCACTGCCAAAGAGTTTCTT) and nested upstream primer Vγint (CTCGAGTGCGCTGCCTACAGAGAGG) or Vγ9.1 for 15 cycles under similar conditions. If insufficient product was present after the second round of PCR, the second round was performed for 25 cycles. All PCR products were analyzed by gel electrophoresis through 1.2% agarose to demonstrate successful amplification and homogeneity of the product. To check for possible contamination by spurious DNA templates, reactions without addition of DNA were performed with each experiment. These control reactions consistently failed to show any product detectable by agarose gel electrophoresis. PCR products were precipitated with ethanol and 50% of each preparation was analyzed by DGGE as described [8,9]. Briefly, samples were denatured at 95°C and reannealed at 60°C. Electrophoresis was performed at 60°C for 6 h at 150 V through 6.5% polyacrylamide containing an increasing linear gradient from 2.45 M urea/14% formamide to 3.85 M urea/22% formamide. Gels were stained with ethidium bromide and photographed under UV transillumination.

PCR/RPA An RNase protection assay for the detection of specific clonal TCR-γ rearrangements was performed as described and illustrated previously [10]. Briefly, a tumor-specific RNA probe was generated by PCR amplification of DNA from specimens histopathologically diagnostic for CTCL and showing the strongest clonal band pattern by PCR/DGGE analysis. This was accomplished using downstream primer JγT7 (GCTAATACGACTCACTATGGGAGAGACAACAAGTGTTGTTCCAC) and upstream primers Vγ1.3 (CGGCTACATCCACTGGTACCT) or Vγ9.4 (CGCCTGGAATGTGTGGTGTCT). The sequence of the promoter of T7 RNA polymerase is underlined [19]. Amplifications were carried out in a volume of 50 μl for 30 cycles with an annealing temperature of 60°C. Other PCR conditions were similar to those noted above for PCR/DGGE. Ten percent of this PCR product was transcribed *in vitro* using a transcription kit (Stratagene, La Jolla, CA) with 10 U of T7 RNA polymerase and 2 mCi/ml of α-^{32}P-UTP (800 Ci/mmol; Dupont, NEN, Boston, MA) in the presence of unlabeled ATP, CTP, and GTP according to the instructions of the manufacturer. Test RNA was similarly synthesized by PCR amplification using down-stream primer Jγ1.7 (GTTCCACTGCCAAAGAGTTTCTT) and upstream primers VγT7 (GCTAATACGACTCACTATGGGAGACGGCTACATCCACTGGTACCT) or Vγ9T7 (GCTAATACGACTCACTATGGGAGACGCCTGGAATGTGTGGTGTCT) under otherwise identical conditions, and subsequent transcription of 20% of this product with 20 U T7 RNA polymerase in the presence of unlabeled NTPs. After transcription, the DNA template was digested with 40 U RNase-free DNase I (Boehringer Mannheim, Germany). Two to five percent of the RNA probe was hybridized to 20% of each test RNA overnight at 64°C in 85% formamide/1 mM ethylenediaminetetraacetic acid/40 mM piperazine N-N′-bis:2-ethane sulfonic acid (PIPES; pH 5.6). The resulting RNA hybrids were subjected to digestion with 40 μg/ml RNase A (Boehringer Mannheim) at

40–42°C for 30 min, precipitated with ethanol, and analyzed for 2 h at 45 V/cm by gel electrophoresis through 6% polyacrylamide/7.6 M urea. Gels were exposed to XAR 5 film (Kodak, Rochester, NY) overnight at −70°C with an intensifying screen. Visual inspection was used to perform semiquantitative assessment of PCR/RPA band intensities in serial dilutions of diagnostic test RNA into normal blood control DNA.

RESULTS

Detection of Dominant Clonality Using PCR/DGGE In a first step toward molecular staging of the disease, PCR/DGGE was performed with DNA extracted from every biopsy of each CTCL patient. As denaturing gradient gels separate DNA fragments based on their precise nucleotide sequence in addition to their size [20], PCR products of TCR-γ gene rearrangements present in a polyclonal population of cells form a smear over the length of the gel, whereas a clonal fraction of at least 0.1–1% of cells with a rearranged TCR-γ gene results in one or more distinct bands [8,9]. Clonal bands were considered valid when the same pattern of bands was detected either in more than one biopsy specimen from the same patient or in at least two independent PCR amplifications of DNA from the same site. **Table I** shows the results of PCR/DGGE analysis in comparison to the histopathologic evaluation. In six patients, detection of clonality by PCR/DGGE correlated with the result of microscopic examination. However, PCR/DGGE clearly demonstrated the presence of the same clonal population of cells in four different biopsy specimens from patient E that were deemed uninvolved by morphologic criteria. In contrast, PCR/DGGE failed to demonstrate unequivocally lymphomatous involvement of a lymph node that histologically was considered positive for the presence of the tumor (patient A) as well as in several other tissue samples that were histologically uninvolved by CTCL.

Generation of Tumor-Specific RNA Probes In the next set of analyses, a tumor-specific, radiolabeled RNA probe was generated for each case. DNA from the biopsy showing the strongest clonal band by PCR/DGGE was amplified with the primers JγT7 and Vγ1.3, followed by *in vitro* transcription in the presence of ^{32}P-labeled UTP. The most intense clonal bands detected by PCR/DGGE were found in skin lesions in seven of the eight patients. Only the tumor-specific RNA probe of patient C was synthesized from a lymph node biopsy, because using histology and PCR/DGGE, the skin lesions of this patient appeared to be in remission at the time of the biopsy after electron beam radiation therapy.

Molecular Staging Using PCR/RPA The tumor-specific RNA probe of each patient was hybridized to test RNA generated from each of the other available biopsies from that patient and from logarithmic dilutions of DNA extracted from the diagnostic site into normal peripheral blood DNA obtained from healthy volunteers. After hybridization, the resulting RNA duplexes were subjected to digestion with RNase A, followed by polyacrylamide gel electrophoresis under denaturing conditions and autoradiography. Test RNA synthesized from peripheral blood of four normal individuals was analyzed in parallel in each assay to exclude false-positive results. In prior studies using PCR/RPA, false positives were also excluded using normal bone-marrow controls [10]. As shown in **Table I**, PCR/RPA detected the respective tumor-specific TCR-γ gene rearrangement in every available biopsy specimen from each of the patients in this series. These results indicate a widespread dissemination of the disease not only in patients with known extracutaneous lesions, but possibly also in a substantial number of patients in stage I at the time of diagnosis.

Patient H, who was in stage I, had a few morphologically abnormal cells in his peripheral blood without fulfilling the criteria for blood involvement [11]. PCR/DGGE failed to demonstrate a clonal pattern unequivocally. PCR/RPA, however, clearly revealed circulating cells of the malignant clone. **Figure 1** shows the analysis of biopsies from patient G who was also in stage I. An aliquot of the RNA probe of this case that has not been subjected to digestion with RNase A has a clonal band length of approxi-

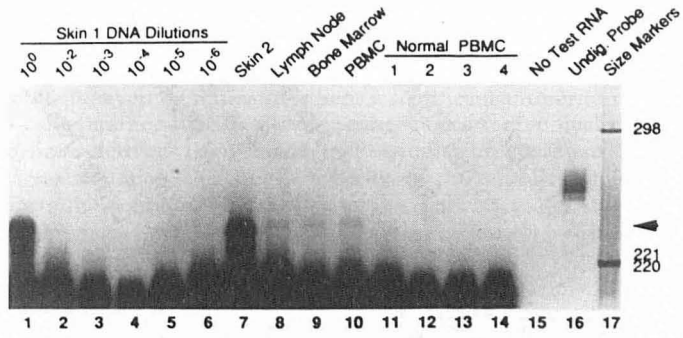

Figure 1. PCR/RPA demonstrates systemic involvement by CTCL in stage I disease. RNase protection assay of DNA extracted from biopsies of patient G and normal peripheral blood mononuclear cells using an RNA probe derived from a diagnostic skin biopsy specimen. For quantitation of the involvement of the nondiagnostic biopsies, logarithmic dilutions of DNA from the diagnostic biopsy into normal PBMC DNA were analyzed in parallel. The position of the tumor-specific protected band is indicated by the *arrow*. The size markers are ^{35}S-labeled *Hinf*I/*Eco*RI restriction fragments of the plasmid pBR322.

mately 251 base pairs (lane 16). Also present in this lane is a smear that presumably stems from reactive lymphocytes found in the lesional tissue. As the test RNA transcribed from a PCR product amplified with primers VγT7 and JγI7 is shorter than the RNA probe, probe molecules that have been protected from RNase cleavage by perfectly matched test RNA generated from DNA of cells of the CTCL clone have a length of 232 base pairs [10]. Apart from the diagnostic sample in lane 1, a band is detectable in this position in lanes 7–10, indicating the presence of the tumor in a second skin biopsy, peripheral blood, bone marrow, and a lymph node, although the patient was clinically and pathologically in stage IA. The same band is also present in a dilution of 10^{-2} of the DNA from the diagnostic specimen into normal peripheral blood DNA.

This limiting dilution step appears considerably lower than the sensitivity of 10^{-5} that has been reported for PCR/RPA in the analysis of acute lymphoblastic leukemia [10]. The most likely explanation for the difference is that, in contrast to diagnostic bone-marrow aspirates of acute leukemias, the malignant cell clone frequently comprises only a minor fraction of the total cell population in a typical lesion of CTCL [21]. This is due to an often large number of reactive lymphocytes and the invariable presence of keratinocytes, fibroblasts, and other stromal cells in skin biopsies. Consequently, the tumor-specific gene rearrangement may be diluted out earlier than it would have been using a relatively homogeneous tumor cell population.

Findings Suggestive of Oligoclonality in Some CTCL Cases A discrepancy between the molecular assays and light microscopy was noted in patient F. Although lymphomatous involvement of a lymph node was diagnosed histologically, PCR/RPA performed using the clonal VγI-rearrangement demonstrated a tumor-specific band only at the low intensity of 1:5000, whereas PCR/DGGE did not detect a clonal band. This finding is in contrast to the analyses of other patients, where the maximum sensitivity of morphologic examination was found to correspond to a limiting dilution in PCR/RPA of 1:500 (patient A) or higher. Therefore, we decided to study the clonal TCR-γ gene rearrangement containing the Vγ9 gene segment that had been found in the diagnostic skin specimen of this patient [9]. Surprisingly, PCR/DGGE readily detected the same clonal Vγ9 rearrangement in both the skin and lymph node biopsies **(Fig 2)**. PCR/RPA performed with the primer set for Vγ9 rearrangements showed a strong tumor-specific band with only slightly diminished intensity when compared to the diagnostic sample **(Fig 3)**.

Two explanations can be offered for this finding. First, as the normal polyclonal rearrangements compete with the tumor-specific rearrangements during the PCR reaction, a clonal Vγ9 rearrange-

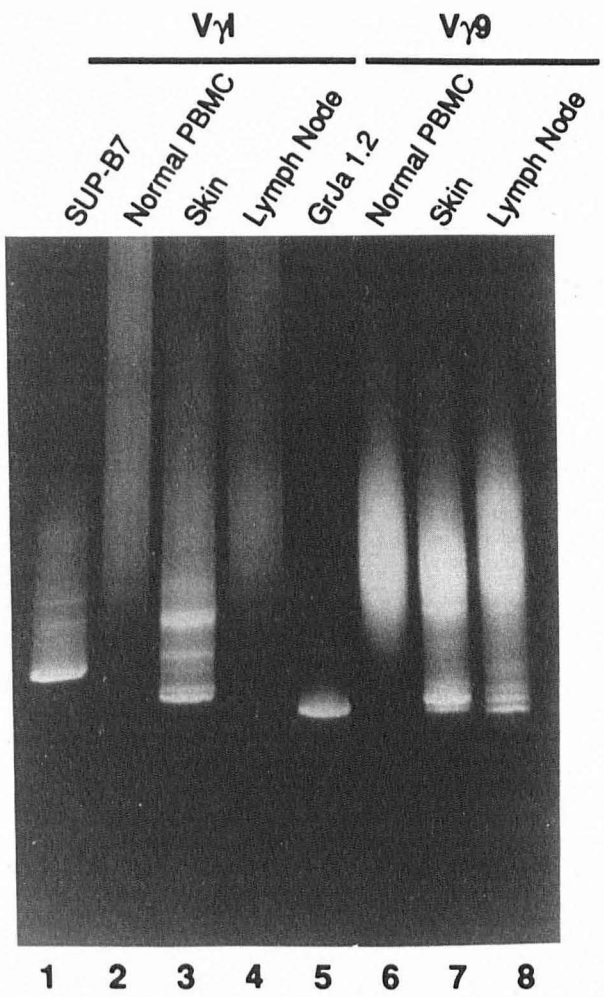

Figure 2. PCR/DGGE detects clonal T-cell populations in diagnostic skin and lymph node biopsy specimens from CTCL patient F. DNA extracted from both biopsies was amplified with the primer pairs specific for Vγ1 and Vγ9 TCR-γ gene rearrangements. The clonal control lanes are DNA from the lymphoblastoid cell line SUP-B7 for Vγ1 and an amplified Vγ9 TCR-γ gene rearrangement cloned into the phage M13mp18 (GrJa1.2). The polyclonal control lanes contain DNA from normal PBMC for both primer combinations.

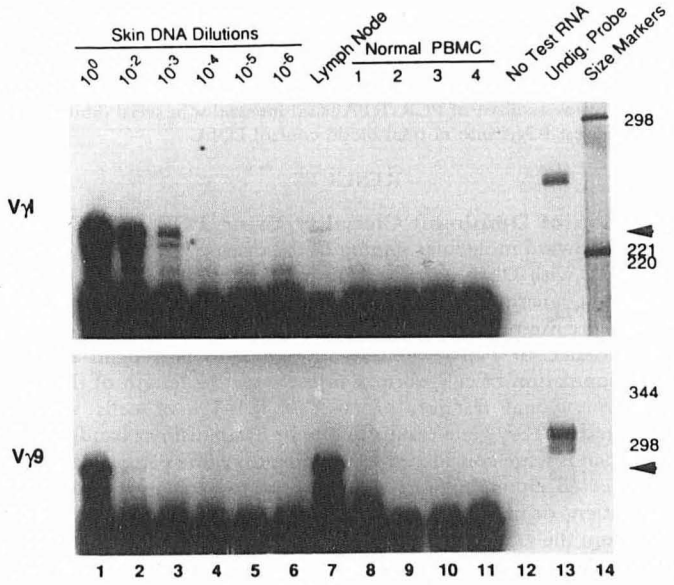

Figure 3. PCR/RPA detects clonal T-cell populations in diagnostic skin and lymph node biopsy specimens from CTCL patient F. DNA extracted from both biopsy specimens of patient F and normal PBMC was analyzed using RNA probes derived from the clonal Vγ1 and Vγ9 rearrangements of the diagnostic skin specimen of patient F. For quantitation of the involvement of the lymph node biopsy, logarithmic dilutions of DNA from the diagnostic skin biopsy into normal PBMC DNA were analyzed in parallel. The position of the respective tumor-specific protected band is indicated by the *arrows*.

ment may be amplified better than a clonal Vγ1 rearrangement and ultimately give a stronger signal because rearrangements involving the Vγ9 gene segment are generally less frequent than rearrangements involving the nine members of the Vγ1 subgroup. Second, the tumor of this patient may consist of two malignant clones of lymphocytes, each distinguished by a different type of TCR-γ gene rearrangement. Although both clones may be equally represented in the skin biopsy, the clone carrying the rearrangement utilizing Vγ9 could have spread preferentially to the biopsied lymph node.

The tumor of patient D represents another example of CTCL with two possible clonal cell populations. Clonality was detected in the diagnostic lesion for Vγ1 and Vγ9 by PCR/DGGE [9]. When the PCR primers for Vγ1 were used to generate the RNA probe for RPA analysis, the undigested probe clearly consisted of two separate bands of approximately equal intensity (not shown). These findings indicate the presence of two distinct Vγ1 TCR-γ gene rearrangements and at least one rearrangement involving the Vγ9 segment in this tumor.

DISCUSSION

This study demonstrates the use of a sensitive and tumor-specific molecular staging procedure for CTCL. A combination of PCR amplification of rearranged TCR-γ genes and RNase protection assay was found to be considerably more sensitive than histopathologic examination for determining the extent of the disease. The malignant T-cell clone could be detected by PCR/RPA in all available biopsy specimens from six patients beyond stage I. These data support the concept of CTCL as a systemic disease and suggest that any potentially curative therapy in advanced-stage CTCL would have to be based on a systemic approach.

In addition, malignant cells were also found in extracutaneous specimens of two patients considered to be in stage I. This finding is of special interest because good control of disease, sometimes including complete remissions of long duration, can be achieved in the majority of this group of patients by a variety of treatment options targeted at the skin. These therapies include topical mechlorethamine, electron beam radiotherapy, or combined administration of psoralen and irradiation with UVA light (PUVA) [11,22,23]. Further studies are indicated to determine whether the detection of extracutaneous disease with highly sensitive diagnostic techniques at this stage correlates with the clinical response to therapy. However, prognosis in CTCL is related to conventional clinicopathologic staging [11]. It has not yet been established whether the detection of the tumor clone in histologically uninvolved extracutaneous tissues has any prognostic relevance in this disease. Nevertheless, molecular staging could potentially help to define prognostic subgroups more accurately than previously possible.

Apart from detecting lymphomatous involvement in histopathologically normal tissues, our data also show that PCR/RPA has superior sensitivity when compared to PCR/DGGE. However, this fact does not diminish the usefulness of PCR/DGGE because each test has been designed to address somewhat different diagnostic problems. The major application of PCR/DGGE is likely to be the primary diagnosis of malignancy based on the demonstration of clonality among lymphocytes, particularly when a relatively small number of lymphocytes is available for analysis [9]. In contrast, the PCR/RPA technique was developed as a staging tool to detect the dissemination or persistence of a particular malignant lymphoid

clone with extreme sensitivity in patients with a proved lymphoid malignancy [10]. In fact, PCR/RPA requires a diagnostic reference tissue for construction of probes used in the procedure. In the current study, the selection of this reference tissue was based on PCR/DGGE results. Therefore, both techniques complement each other for different steps in the diagnosis and staging of a lymphoma rather than represent alternative approaches for the same problem. Extensive studies involving a variety of inflammatory skin disease controls will be required before it can be determined whether PCR/RPA can be used reliably to identify occult neoplastic T-cell clones in cases of CTCL that are so early that they fail to show dominant clonality by PCR/DGGE.

Prior reports have described the tumor-specific detection of circulating malignant cells in Sezary syndrome, a systemic subtype of CTCL characterized by generalized erythroderma and large numbers of atypical lymphocytes in the peripheral blood [11]. These approaches relied on PCR amplification of the TCR-β chain gene rearrangement of the individual tumors [24,25]. As a neoplasm of the mature T-cell type, virtually all cases of CTCL can be expected to carry and express a rearranged TCR-β gene [11,12]. However, the TCR-β locus comprises at least 24 Vβ subgroups [4]. This complexity makes the choice of oligonucleotide primers for a successful PCR amplification of TCR-β gene rearrangements difficult and necessitates the use of RNA as the starting material. PCR amplification of TCR-β gene rearrangements has been accomplished by the use of a panel of primers specific for each Vβ gene family [26]. To avoid performing multiple amplification reactions with a panel of Vβ-specific primers, one group has described the use of a single, highly degenerate primer in conjunction with a Cβ-primer to amplify rearrangements involving 18 Vβ gene families [24]. Under these conditions, however, the PCR product appears to be rather impure and to contain a variety of nonhomologous fragments [24]. The other report involved the use of a specifically designed multiprobe RNase protection assay as an additional preliminary analytic step to identify the correct Vβ family prior to PCR amplification [25]. In both of these approaches, the precise base composition of the V-D-J junction of the clonotypic rearrangement is determined by cloning of the PCR product and DNA sequencing. In the next step, an oligonucleotide probe specific for the junctional region is chemically synthesized to facilitate detection of tumor cells by subsequent PCR amplification. Both approaches were able to demonstrate circulating tumor cells in Sezary syndrome. The former method has also been used to detect tumor cells in histologically uninvolved skin and lymph node but not blood obtained from one patient with mycosis fungoides [Lessin SR, DiLosa RM, Benoit B, Jaworsky C, Rovera G: Molecular detection of malignant infiltration in uninvolved skin and lymph node in a patient with cutaneous T-cell lymphoma (CTCL) (abstr). *J Invest Dermatol* 96:535, 1991].

In contrast to these methods, the molecular assays used in this report assess TCR-γ rather than TCR-β gene rearrangements. Although the TCR-γ gene is rarely expressed, it nevertheless has undergone rearrangement in the overwhelming majority of CTCL cases [9,27]. We chose to study TCR-γ gene rearrangements as diagnostic markers for these tumors because the comparatively simple structure of this locus makes it particularly suitable for PCR-based analyses [4,5]. Both the PCR/DGGE and PCR/RPA techniques are routinely performed with only two sets of PCR primers. These primer combinations permit the analysis of TCR-γ gene rearrangements involving most of the Vγ gene segments known to undergo genetic recombination [16–18]. From our experience, over 90% of all TCR-γ gene rearrangements are amplifiable with this approach [9]. Rare rearrangements involving the remaining Vγ genes can be amplified using two additional Vγ-specific PCR primers (unpublished results). Apart from the simplicity of the PCR amplification, PCR/RPA has the additional important advantage that cloning of the PCR products, DNA sequencing and chemical oligonucleotide synthesis are not required for each individual case. Moreover, the initial substrate for analysis is DNA rather than RNA. RNA is less stable and may be less reliably isolated from biopsy specimens.

This study demonstrates the applicability of the PCR/RPA technique even in situations where a significant proportion of the so-called tumor-specific RNA probe consists of polyclonal sequences derived from reactive T lymphocytes present in the diagnostic lesion. Although impurity of the probe undoubtedly affects the sensitivity of the technique, earlier studies show that non-homogeneity of the probe does not reduce the sensitivity of PCR/RPA as much as may be anticipated. For example, a clonal population representing only 0.1% of the total cells in bone marrow could still be used to generate a probe that detected a 1:1000 dilution of DNA from that clone in DNA from polyclonal cells [10]. It may be possible to enhance the sensitivity of the technique by amplifying the probe from DNA of TCR-γ gene rearrangements purified from the diagnostic specimen. This could be accomplished fairly easily by extracting the clonal bands from DGGE gels. In any event, as suggested by our data in this report, maximal sensitivity of the technique may not be necessary to find disseminated CTCL in most cases.

In our study, CTCL was used as a model disease for the sensitive molecular staging of malignant lymphomas. The results demonstrate that PCR/RPA is readily applicable for lymphomas carrying TCR-γ gene rearrangements. Furthermore, PCR/RPA can be modified for analysis of rearrangements of the immunoglobulin heavy chain gene (unpublished results). This application is likely to extend the use of this assay to most lymphomas of B cell lineage, which comprise the majority of all lymphoid neoplasms [1]. As in CTCL, a sensitive molecular-staging procedure such as PCR/RPA could potentially improve the definition of prognostic subgroups in other lymphoproliferative processes. Furthermore, the availability of more accurate staging could eventually improve the selection of appropriate treatment for patients with potentially curable conditions such as large-cell lymphoma. PCR-based assays might also allow a more accurate monitoring of individual lymphoma patients during remission to identify patients who might benefit from additional therapy. Finally, these assays could be useful in treatment regimens involving transplanting with autologous bone marrow or peripheral bone-marrow stem cells by permitting screening of grafts for occult malignancy.

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